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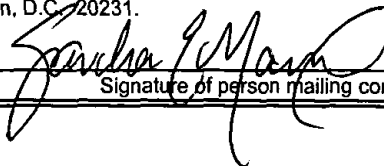
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APPLICATION
FOR
UNITED STATES LETTERS PATENT

APPLICANT : Richard F Selden, Douglas Treco & Michael W. Heartlein
TITLE : IN VIVO PRODUCTION AND DELIVERY OF
ERYTHROPOIETIN OR INSULINOTROPIN FOR GENE
THERAPY

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IN VIVO PRODUCTION AND DELIVERY OF
ERYTHROPOIETIN OR INSULINOTROPIN
FOR GENE THERAPY

Description

05 Related Applications

Dr. Sa
ai ~~This application~~ is a continuation-in-part of U. S. Serial No. 07/787,840, filed November 5, 1991, entitled "In Vivo Protein Production and Delivery System for Gene Therapy" and of U. S. Serial No. 07/789,188, filed November 5, 1991, entitled "Targeted Introduction of DNA Into Primary or Secondary Cells and Their Use for Gene Therapy". The teachings of these applications are incorporated by reference.

Background of the Invention

15 A variety of congenital, acquired, or induced syndromes are associated with insufficient numbers of erythrocytes (red blood cells or RBCs). The clinical consequence of such syndromes, collectively known as the anemias, is a decreased oxygen-carrying potential of the blood, resulting in fatigue, weakness, and failure-to-thrive. Erythropoietin (EPO), a glycoprotein of molecular mass 34,000 daltons, is synthesized and released into the systemic circulation in response to reduced oxygen tension in the blood. EPO, primarily synthesized in the kidney and, to a lesser extent, in the liver, acts on erythroid precursor cells [Colony Forming Units-Erythroid (CFU-E) and Burst-Forming Units-Erythroid (BFU-E)] to promote differentiation into reticulocytes and, ultimately, mature erythrocytes.

15 Purified human EPO or recombinant human EPO may be administered to patients in order to alleviate anemia by increasing erythrocyte production. Typically, the protein is administered by regular intravenous injections. The administration of EPO by injection is an
20 imperfect treatment. Normal individuals maintain a relatively constant level of EPO, which is in the range of 6-30 mU/ml, depending on the assay used. After typical treatment regimens, serum EPO levels may reach 3,000-5,000 mU/Ml following a single injection, with
25 levels falling over time as the protein is cleared from the blood.

If a relatively constant level of EPO is to be provided in the blood (i.e., to mimic the normal physiology of the protein), a delivery system that is capable of releasing a continuous, precisely dosed quantity of EPO into the blood is necessary.

Summary of the Invention

The present invention relates to transfected primary and secondary somatic cells of vertebrate origin, particularly mammalian origin, transfected with exogenous genetic material (DNA or RNA) which encodes a clinically useful product, such as erythropoietin (EPO) or insulinotropin [e.g. derivatives of glucagon-like peptide 1 (GLP-1) such as GLP(7-37), GLP(7-36), GLP-1(7-35) and GLP-1(7-34) as well as their carboxy-terminal amidated derivatives produced by in vivo amidating enzymes and derivatives which have amino acid alterations or other alterations which result in substantially the same biological activity or stability in the blood as that of a truncated GLP-1 or enhanced biological activity or stability], methods by which primary and secondary cells are transfected to include exogenous genetic material encoding EPO or insulinotropin, methods of producing clonal cell strains or heterogenous cell strains which express exogenous genetic material encoding EPO or insulinotropin, a method of providing EPO or insulinotropin in physiologically useful quantities to an individual in need thereof, through the use of transfected cells of the present invention or by direct injection of DNA encoding EPO into an individual; and methods of producing antibodies against the encoded product using the transfected primary or secondary cells. Transfected cells containing EPO-encoding exogenous genetic material express EPO and, thus, are useful for preventing or treating conditions in which EPO production and/or utilization are inadequate or compromised, such as in any condition or disease in which there is anemia.

Similarly, transfected cells containing insulinotropin-encoding exogenous genetic material express insulinotropin and, thus, are useful for treating individuals in whom insulin secretion, sensitivity or function is
05 compromised (e.g., individuals with insulin-dependent or non-insulin dependent diabetes).

The present invention includes primary and secondary somatic cells, such as fibroblasts, keratinocytes, epithelial cells, endothelial cells, glial cells, neural
10 cells, formed elements of the blood, muscle cells, other somatic cells which can be cultured and somatic cell precursors, which have been transfected with exogenous DNA encoding EPO or exogenous DNA encoding insulinotropin. The exogenous DNA is stably integrated into the
15 cell genome or is expressed in the cells episomally. The exogenous DNA encoding EPO is introduced into cells operatively linked with additional DNA sequences sufficient for expression of EPO in transfected cells. The exogenous DNA encoding EPO is preferably DNA encoding
20 human EPO but, in some instances, can be DNA encoding mammalian EPO of non-human origin. EPO produced by the cells is secreted from the cells and, thus, made available for preventing or treating a condition or disease (e.g., anemia) in which EPO production and/or
25 utilization is less than normal or inadequate for maintaining a suitable level of RBCs. Cells produced by the present method can be introduced into an animal, such as a human, in need of EPO and EPO produced in the cells is secreted into the systemic circulation. As a result,
30 EPO is made available for prevention or treatment of a condition in which EPO production and/or utilization is

less than normal or inadequate to maintain a suitable level of RBCs in the individual. Similarly, exogenous DNA encoding insulintropin is introduced into cells operatively linked with additional DNA sequences

05 sufficient for expression of insulintropin in transfected cells. The encoded insulintropin is made available to prevent or treat a condition in which insulin production or function is compromised or glucagon release from the pancreas is to be inhibited.

10 Primary and secondary cells transfected by the subject method can be seen to fall into three types or categories: 1) cells which do not, as obtained, produce and/or secrete the encoded protein (e.g., EPO, insulintropin; 2) cells which produce and/or secrete the encoded
15 protein (e.g., EPO, insulintropin) but in lower quantities than normal (in quantities less than the physiologically normal lower level) or in defective form, and 3) cells which make the encoded protein (e.g., EPO or insulintropin) at physiologically normal levels, but are
20 to be augmented or enhanced in their production and/or secretion of the encoded protein.

Exogenous DNA encoding EPO is introduced into primary or secondary cells by a variety of techniques. For example, a construct which includes exogenous DNA
25 encoding EPO and additional DNA sequences necessary for expression of EPO in recipient cells is introduced into primary or secondary cells by electroporation, microinjection, or other means (e.g., calcium phosphate precipitation, modified calcium phosphate precipitation,
30 polybrene precipitation, microprojectile bombardment, liposome fusion, receptor-mediated DNA delivery).

Transfected cells of the present invention are useful, as populations of transfected primary cells, 20 transfected clonal cell strains, transfected heterogenous cell strains, and as cell mixtures in which at least one representative cell of one of the three preceding categories of transfected cells is present, as a delivery system for treating an individual with a condition or 25 disease which responds to delivery of EPO (e.g. anemia) or for preventing the development of such a condition or disease. In the method of the present invention of providing EPO, transfected primary cells, clonal cell strains, or heterogenous cell strains, are administered 30 to an individual in need of EPO, in sufficient quantity and by an appropriate route, to deliver EPO to the

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Clonal cell strains of transfected secondary cells (referred to as transfected clonal cell strains) expressing exogenous DNA encoding EPO (and, optionally, including a selectable marker gene) are produced by the method of the present invention. The present method includes the steps of: 1) providing a population of primary cells, obtained from the individual to whom the transfected primary cells will be administered or from another source; 2) introducing into the primary cells or into secondary cells derived from primary cells a DNA construct which includes exogenous DNA encoding EPO and additional DNA sequences necessary for expression of EPO, thus producing transfected primary or secondary cells; 3) maintaining transfected primary or secondary cells under conditions appropriate for their propagation; 4) identifying a transfected primary or secondary cell; and 5) producing a colony from the transfected primary or secondary cell identified in (4) by maintaining it under

appropriate culture conditions and for sufficient time for its propagation, thereby producing a cell strain derived from the (founder) cell identified in (4). In one embodiment of the method, exogenous DNA encoding EPO
05 is introduced into genomic DNA by homologous recombination between DNA sequences present in the DNA construct used to transfect the recipient cells and the recipient cell's genomic DNA. Clonal cell strains of transfected secondary cells expressing exogenous DNA encoding insulinotropin (and, optionally, including a selectable
10 marker gene) are also produced by the present method.

In one embodiment of the present method of producing a clonal population of transfected secondary cells, a cell suspension containing primary or secondary cells is
15 combined with exogenous DNA encoding EPO and DNA encoding a selectable marker, such as the bacterial neo gene. The two DNA sequences are present on the same DNA construct or on two separate DNA constructs. The resulting combination is subjected to electroporation, generally at
20 250-300 volts with a capacitance of 960 μ Farads and an appropriate time constant (e.g., 14 to 20 msec) for cells to take up the DNA construct. In an alternative embodiment, microinjection is used to introduce the DNA construct containing EPO-encoding DNA into primary or
25 secondary cells. In either embodiment, introduction of the exogenous DNA results in production of transfected primary or secondary cells. Using the same approach, electroporation or microinjection is used to produce a clonal population of transfected secondary cells containing
30 exogenous DNA encoding insulinotropin alone, or insulinotropin and a selectable marker.

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The present invention is particularly advantageous in treating anemia and other conditions in which EPO production, utilization or both is compromised in that it: 1) makes it possible for one gene therapy treatment, 05 when necessary, to last a patient's lifetime; 2) allows precise dosing (the patient's cells continuously determine and deliver the optimal dose of EPO based on physiologic demands, and the stably transfected cell strains can be characterized extensively in vitro prior to 10 implantation, leading to accurate predictions of long term function in vivo); 3) is simple to apply in treating patients; 4) eliminates issues concerning patient compliance (periodic administration of EPO is no longer necessary); and 5) reduces treatment costs (since the 15 therapeutic protein is synthesized by the patient's own cells, investment in costly protein production and purification facilities is unnecessary).

Brief Description of the Drawings

Figure 1 is a schematic representation of plasmid 20 pXEP01. The solid black arc represents the pUC12 backbone and the arrow denotes the direction of transcription of the ampicillin resistance gene. The stippled arc represents the mouse metallothionein promoter (pmMT1). The unfilled arc interrupted by black 25 boxes represents the human erythropoietin EPO gene (the black boxes denote exons and the arrow indicates the direction hEPO transcription). The relative positions of restriction endonuclease recognition sites are indicated.

Figure 2 is a schematic representation of plasmid 30 pCDNEO. This plasmid has the neo gene from plasmid

pSV2neo (a BamHI-BglIII fragment) inserted into the BamHI site of plasmid pcD; the amp and pBR322ori sequences are from pBR322; the polyA, 19S splice junction, and early promoter sequences are from SV40.

05 Figure 3 is a schematic representation of plasmid pXGH301. This plasmid contains both the human growth hormone (hGH) and neo resistance genes. Arrows indicate the directions of transcription of the various genes. The positions of restriction endonuclease recognition sites, the mouse metallothionein promoter (pMMT1), the
10 amp resistance gene, and the SV40 early promoter (pSV40 early) are indicated.

 Figure 4 is a schematic representation of plasmid pE3neoEPO. The positions of the human erythropoietin
15 gene and the neo and amp resistance genes are indicated. Arrows indicate the directions of transcription of the various genes. pMMT1 denotes the mouse metallothionein promoter (driving hEPO expression) and pTK denotes the Herpes Simplex Virus thymidine kinase promoter (driving
20 neo expression). The dotted regions of the map mark the positions of human HGPRT sequences. The relative positions of restriction endonuclease recognition sites are indicated.

 Figure 5A shows results of Western blot analysis of
25 hEPO secreted by normal human fibroblasts cotransfected with pXEPO1 and pcDNEO. The left panel shows the Western analysis and the right panel shows a photograph of the Coomassie blue stained gel. Lanes C,E, and M signify Control sample (supernatant from untransfected human
30 fibroblasts), Experimental sample (supernatant from a

clonal strain of human fibroblasts expressing hEPO), and marker lanes, respectively.

Figure 5B shows results of Western blot analysis of hEPO secreted by normal human fibroblasts cotransfected
05 with pXEPO1 and pcDNEO. Supernatant from a clonal strain of human fibroblasts expressing hEPO (lane 1) was further analyzed for glycosylation by treatment with endoglycosidase-F (lane 2), neuraminidase (lane 3), and O-glycanase (lane 4).

10 Figure 6A shows results of an assay to detect hEPO in the serum of mice implanted with transfected rabbit fibroblasts expressing hEPO.

Figure 6B shows hematocrit (HCT) levels in control mice and mice implanted with transfected rabbit fibro-
15 blasts expressing hEPO.

Detailed Description of the Invention

The present invention relates to the use of genetically engineered cells to deliver a clinically useful or otherwise desirable substance to an individual in whom
20 production of the substance is desired (e.g., to prevent or treat a disease or condition in which the product is produced or functions at an unacceptable level). In particular, it relates to the use of genetically engineered cells to deliver EPO to the systemic circulation
25 of an individual in need of EPO, resulting in an increase in mature red blood cell numbers, an increase in the oxygen-carrying potential of the blood, and an alleviation of the symptoms of anemia. The present invention provides a means of delivering EPO at physiologically

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defined as a cell strain that is derived from two or more founder cells.

As described herein, primary or secondary cells of vertebrate, particularly mammalian, origin have been
05 transfected with exogenous DNA encoding EPO and shown to produce the encoded EPO reproducibly, both in vitro and in vivo, over extended periods of time. In addition, the transfected primary and secondary cells have been shown to express EPO in vivo at physiologically relevant
10 levels. The EPO expressed has been shown to have the glycosylation pattern typical of EPO purified from human urine or recombinant human EPO. This demonstration is in sharp contrast to what one of skill in the art would predict, since, for example, even experts in the field
15 see the finite life span of normal somatic cells and the inability to isolate or grow the relevant transplantable cells as precluding their use for gene therapy unless the cells are genetically modified using retroviruses (Miller, A.D., Blood, 76:271-278 (1990)). However, the
20 transplantation of retrovirally treated fibroblasts has been shown to provide, at best, only transient metabolic improvements, and is seen to have serious limitations as a therapeutic system. In addition, until Applicants' work, this had not been done for EPO. Normal (non
25 immortal) fibroblasts are characterized as being "much more difficult to transfect than continuous cell lines by using calcium phosphate precipitation techniques." (Miller, A.D., Blood, 76:271-278 (1990)). Furthermore, in considering non-retroviral techniques for gene
30 therapy, it is typical of experts in the field to believe "...the efficiency of gene delivery is dismal...A

05 Surprisingly, Applicants have been able to produce transfected primary and secondary cells which include exogenous DNA encoding EPO and express the exogenous DNA.

As also described herein, it is possible to transfect primary or secondary cells of vertebrate, particularly mammalian, origin with exogenous DNA encoding insulinotropin and to use them to provide insulinotropin

30 larly mammalian, origin with exogenous DNA encoding
insulinotropin and to use them to provide insulinotropin

to an individual in whom insulin production, function and/or sensitivity is compromised.

Transfected Cells

Primary and secondary cells to be transfected in order to produce EPO or insulinotropin can be obtained from a variety of tissues and include all cell types which can be maintained and propagated in culture. For example, primary and secondary cells which can be transfected by the present method include fibroblasts, keratinocytes, epithelial cells (e.g., mammary epithelial cells, intestinal epithelial cells), endothelial cells, glial cells, neural cells, formed elements of the blood (e.g., lymphocytes, bone marrow cells), muscle cells, other somatic cells which can be cultured, and precursors of these somatic cell types. Primary cells are preferably obtained from the individual to whom the transfected primary or secondary cells are administered. However, primary cells may be obtained from a donor (other than the recipient) of the same species or another species (e.g., mouse, rat, rabbit, cat, dog, pig, cow, bird, sheep, goat, horse).

Transfected primary and secondary cells can be produced, with or without phenotypic selection, as described herein, and shown to express exogenous DNA encoding EPO or exogenous DNA encoding insulinotropin.

Exogenous DNA

Exogenous DNA incorporated into primary or secondary cells by the present method is DNA encoding the desired product (e.g., EPO or insulinotropin), a functional or

active portion, or a functional equivalent of EPO or
insulinotropin (a protein which has a different amino
acid sequence from that of EPO but has substantially the
same biological function as EPO, or a protein which has a
05 different amino acid sequence from that of GLP-1 related
peptides but functions biologically as insulinotropin).
The DNA can be obtained from a source in which it occurs
in nature or can be produced, using genetic engineering
techniques or synthetic processes. The DNA encoding EPO
10 or insulinotropin will generally be DNA encoding the
human product (i.e., human EPO or human insulinotropin).
In some cases, however, the DNA can be DNA encoding EPO
or insulinotropin of non-human origin (i.e., DNA obtained
from a non-human source or DNA, produced recombinantly or
15 by synthetic methods, which encodes a non-human EPO or
insulinotropin).

The DNA transfected into primary or secondary cells
can encode EPO alone or EPO and another product, such as
a selectable marker to facilitate selection and identifi-
20 cation of transfected cells. Alternatively, the trans-
fected DNA can encode insulinotropin alone or insulinotro-
pin and another product, such as a selectable marker.
After transfection into primary or secondary cells, the
exogenous DNA is stably incorporated into the recipient
25 cell's genome (along with the additional sequences
present in the DNA construct used), from which it is
expressed or otherwise functions. Alternatively, the
exogenous DNA may exist episomally within the transfected
primary or secondary cells. DNA encoding the desired
30 product can be introduced into cells under the control of
an inducible promoter, with the result that cells

produced or as introduced into an individual do not express the product but can be induced to do so (i.e., production is induced after the transfected cells are produced but before implantation or after implantation).

- 05 DNA encoding the desired product can, of course, be introduced into cells in such a manner that it is expressed upon introduction (i.e., without induction).

Selectable Markers

- 10 A variety of selectable markers can be incorporated into primary or secondary cells. For example, a selectable marker which confers a selectable phenotype such as drug resistance, nutritional auxotrophy, resistance to a cytotoxic agent or expression of a surface protein, can be used. Selectable marker genes which can be used
- 15 include neo, gpt, dhfr, ada, pac, hyg and hisD. The selectable phenotype conferred makes it possible to identify and isolate recipient primary or secondary cells.

DNA Constructs

- 20 DNA constructs, which include exogenous DNA encoding the desired product (e.g., EPO, insulinotropin) and, optionally, DNA encoding a selectable marker, along with additional sequences necessary for expression of the exogenous DNA in recipient primary or secondary cells,
- 25 are used to transfect primary or secondary cells in which the protein (e.g., EPO, insulinotropin) is to be produced. Alternatively, infectious vectors, such as retroviral, herpes, adenovirus, adenovirus-associated,

mumps and poliovirus vectors, can be used for this purpose.

05 A DNA construct which includes the exogenous DNA encoding EPO and additional sequences, such as sequences necessary for expression of EPO, can be used (e.g., plasmid pXEPO1; see Figure 1). A DNA construct can include an inducible promoter which controls expression of the exogenous DNA, making inducible expression possible. Optionally, the DNA construct may include a
10 bacterial origin of replication and bacterial antibiotic resistance markers, which allow for large-scale plasmid propagation in bacteria. A DNA construct which includes DNA encoding a selectable marker, along with additional sequences, such as a promoter, polyadenylation site, and
15 splice junctions, can be used to confer a selectable phenotype upon transfected primary or secondary cells (e.g., plasmid pCDNEO). The two DNA constructs are co-transfected into primary or secondary cells, using methods described herein. Alternatively, one DNA con-
20 struct which includes exogenous DNA encoding EPO, a selectable marker gene and additional sequences (e.g., those necessary for expression of the exogenous DNA and for expression of the selectable marker gene) can be used. Such a DNA construct (pE3neoEPO) is described in
25 Figure 4; it includes the EPO gene and the neo gene. Similar constructs, which include exogenous DNA encoding insulinotropin and additional sequences (e.g., sequences necessary for insulinotropin expression) can be produced (e.g., plasmid pXGLP1; see Example 11). These constructs
30 can also include DNA encoding a selectable marker, as

well as other sequences, such as a promoter, a polyadenylation site, and splice junctions.

In those instances in which DNA is injected directly into an individual, such as by injection intomuscles, 5 the DNA construct includes the exogenous DNA and regulatory sequences necessary and sufficient for expression of the encoded product (e.g., EPO) upon entry of the DNA construct into recipient cells.

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10 Transfection of Primary or Secondary Cells and Production of Clonal or Heterogenous Cell Strains

Transfection of cells by the present method is carried out as follows: vertebrate tissue is first obtained; this is carried out using known procedures, such as punch biopsy or other surgical methods of obtain- 15 ing a tissue source of the primary cell type of interest. For example, punch biopsy is used to obtain skin as a source of fibroblasts or keratinocytes. A mixture of primary cells is obtained from the tissue, using known methods, such as enzymatic digestion or explantation. If 20 enzymatic digestion is used, enzymes such as collagenase, hyaluronidase, dispase, pronase, trypsin, elastase and chymotrypsin can be used.

The resulting primary cell mixture can be transfected directly or it can be cultured first, removed from 25 the culture plate and resuspended before transfection is carried out. Primary cells or secondary cells are combined with exogenous DNA encoding EPO, to be stably integrated into their genomes and, optionally, DNA encoding a selectable marker, and treated in order to 30 accomplish transfection. The exogenous DNA and

selectable marker-encoding DNA can each be present on a separate construct (e.g., pXEP01 and pCDNEO, see Figures 1 and 2) or on a single construct (e.g., pE3neoEPO, see Figure 4). An appropriate quantity of DNA to ensure that 5 at least one stably transfected cell containing and appropriately expressing exogenous DNA is produced. In general, 0.1 to 500 μ g DNA is used.

In one embodiment of the present method of producing transfected primary or secondary cells, transfection is 10 effected by electroporation, as described in the Examples. Electroporation is carried out at appropriate voltage and capacitance (and corresponding time constant) to result in entry of the DNA construct(s) into the primary or secondary cells. Electroporation can be 15 carried out over a wide range of voltages (e.g., 50 to 2000 volts) and corresponding capacitance. As described herein, electroporation is very efficient if carried out at an electroporation voltage in the range of 250-300 volts and a capacitance of 960 μ Farads (see Examples 4, 20 5, 7 and 8). Total DNA of approximately 0.1 to 500 μ g is generally used. As described in the Examples, total DNA of 60 μ g and voltage of 250-300 volts with capacitance of 960 μ Farads for a time constant 14-20 of msec. has been used and shown to be efficient.

25 In another embodiment of the present method, primary or secondary cells are transfected using microinjection. See, for instance, Examples 4 and 9. Alternatively, known methods such as calcium phosphate precipitation, modified calcium phosphate precipitation and polybrene 30 precipitation, liposome fusion and receptor-mediated gene delivery can be used to transfect cells. A stably,

transfected cell is isolated and cultured and subcultivated, under culturing conditions and for sufficient time, to propagate the stably transfected secondary cells and produce a clonal cell strain of transfected secondary
5 cells. Alternatively, more than one transfected cell is cultured and subcultured, resulting in production of a heterogenous cell strain.

Transfected primary or secondary cells undergo a sufficient number of doublings to produce either a clonal
10 cell strain or a heterogenous cell strain of sufficient size to provide EPO to an individual in effective amounts. In general, for example, 0.1 cm^2 of skin is biopsied and assumed to contain 100,000 cells; one cell is used to produce a clonal cell strain and undergoes
15 approximately 27 doublings to produce 100 million transfected secondary cells. If a heterogenous cell strain is to be produced from an original transfected population of approximately 100,000 cells, only 10 doublings are needed to produce 100 million transfected cells.

20 The number of required cells in a transfected clonal or heterogenous cell strain is variable and depends on a variety of factors, which include but are not limited to, the use of the transfected cells, the functional level of the exogenous DNA in the transfected cells, the site of
25 implantation of the transfected cells (for example, the number of cells that can be used is limited by the anatomical site of implantation), and the age, surface area, and clinical condition of the patient. To put these factors in perspective, to deliver therapeutic
30 levels of EPO in an otherwise healthy 60 kg patient with anemia, the number of cells needed is approximately the

volume of cells present on the very tip of the patient's thumb.

Episomal Expression of Exogenous DNA

5 DNA sequences that are present within the cell yet do not integrate into the genome are referred to as episomes. Recombinant episomes may be useful in at least three settings: 1) if a given cell type is incapable of stably integrating the exogenous DNA; 2) if a given cell type is adversely affected by the integration of DNA; and
10 3) if a given cell type is capable of improved therapeutic function with an episomal rather than integrated DNA.

Using the transfection and culturing approaches to gene therapy described in Examples 1 and 2, exogenous DNA
15 encoding EPO, in the form of episomes can be introduced into vertebrate primary and secondary cells. Plasmid pE3neoEPO can be converted into such an episome by the addition DNA sequences for the Epstein-Barr virus origin of replication and nuclear antigen [Yates, J.L. Nature
20 319:780-7883 (1985)]. Alternatively, vertebrate autonomously replicating sequences can be introduced into the construct (Weidle, U.H. Gene 73(2):427-437 (1988). These and other episomally derived sequences can also be included in DNA constructs without selectable markers,
25 such as pXEPO1. The episomal exogenous DNA is then introduced into primary or secondary vertebrate cells as described in this application (if a selective marker is included in the episome, a selective agent is used to treat the transfected cells). Similarly, episomal
30 expression of DNA encoding insulinotropin can be

accomplished in vertebrate primary or secondary cells, using the same approach described above with reference to EPO.

Implantation of Clonal Cell Strain or Heterogenous Cell

5 Strain of Transfected Secondary Cells

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The transfected cells produced as described above are introduced into an individual to whom EPO is to be delivered, using known methods. The clonal cell strain or heterogenous cell strain is introduced into an individual, using known methods, using various routes of administration and at various sites (e.g., renal subcapsular, subcutaneous, central nervous system (including intrathecal), intravascular, intrahepatic, intrasplanchnic, intraperitoneal (including intraomental), or intramuscular implantation)]. Once implanted in the individual, the transfected cells produce EPO encoded by the exogenous DNA. For example, an individual who has been diagnosed as anemic is a candidate for a gene therapy cure. The patient has a small skin biopsy performed; this is a simple procedure which can be performed on an out-patient basis. The piece of skin, approximately 0.1 cm², is taken, for example, from under the arm and requires about one minute to remove. The sample is processed, resulting in isolation of the patient's cells (in this case, fibroblasts) and genetically engineered to produce EPO. Based on the age, weight, and clinical condition of the patient, the required number of cells is grown in large-scale culture. The entire process usually requires 4-6 weeks and, at the end of that time, the appropriate number of genetically-engineered cells is

introduced into the individual (e.g., by injecting them back under the patient's skin). The patient is now capable of producing his or her own EPO or additional EPO.

5 Transfected cells, produced as described above, which contain insulinotropin-encoding DNA are delivered into an individual in whom insulin production, secretion, function and/or sensitivity is compromised. They are introduced into the individual by known methods and at
10 various sites of administration (e.g., renal, subcapsular, subcutaneous, central nervous system (including intrathecal), intravascular, intrahepatic, intrasplanchnic, intraperitoneal (including intraomental) or intramuscular implantation). Once implanted in the individual,
15 dual, the transfected cells produce insulinotropin encoded by the exogenous DNA. For example, an individual in whom insulin production, secretion or sensitivity is impaired can receive therapy or preventive treatment through the implantation of transfected cells expressing
20 exogenous DNA encoding insulinotropin produced as described herein. The cells to be genetically engineered are obtained as described above for EPO, processed in a similar manner to produce sufficient numbers of cells, and introduced back into the individual.

25 As this example suggests, the cells used will generally be patient-specific genetically-engineered cells. It is possible, however, to obtain cells from another individual of the same species or from a different species. Use of such cells might require administration of an immunosuppressant, alteration of histocompatibility antigens, or use of a barrier device to prevent rejection of the implanted cells.

In one embodiment, a barrier device is used to prevent rejection of implanted cells obtained from a source other than the recipient (e.g., from another human or from a non-human mammal such as a cow, dog, pig, goat, 5 sheep or rodent). In this embodiment, transfected cells of the present invention are placed within the barrier device, which is made of a material (e.g., a membrane such as Amicon XM-50) which permits the product encoded by the exogenous DNA to pass into the recipient's 10 circulation or tissues but prevents contact between the cells and the recipient's immune system and thus prevents an immune response to (and possible rejection of) the cells by the recipient. Alternatively, DNA encoding EPO or insulinotropin can be introduced into an individual by 15 direct injection, such as into muscle or other appropriate site. In this embodiment, the DNA construct includes exogenous DNA encoding the therapeutic product (e.g., EPO, insulinotropin) and sufficient regulatory sequences for expression of the exogenous DNA in 20 recipient cells. After injection into the individual, the DNA construct is taken up by some of the recipient cells. The DNA can be injected alone or in a formulation which includes a physiologically compatible carrier (e.g., a physiological buffer) and, optionally, other 25 components, such as agents which allow more efficient entry of the DNA construct into cells, stabilize the DNA or protect the DNA from degradation.

Uses of Transfected Primary and Secondary Cells and Cell Strains

Transfected primary or secondary cells or cell strains have wide applicability as a vehicle or delivery system for EPO. The transfected primary or secondary cells of the present invention can be used to administer EPO, which is presently administered by intravenous injection. When transfected primary or secondary cells are used, there is no need for extensive purification of the polypeptide before it is administered to an individual, as is generally necessary with an isolated polypeptide. In addition, transfected primary or secondary cells of the present invention produce the therapeutic product as it would normally be produced.

15 An advantage to the use of transfected primary or secondary cells of the present invention is that by controlling the number of cells introduced into an individual, one can control the amount of EPO. In addition, in some cases, it is possible to remove the transfected cells if there is no longer a need for the product. A further advantage of treatment by use of transfected primary or secondary cells of the present invention is that production can be regulated, such as through the administration of zinc, steroids or an agent which affects transcription of the EPO-encoding DNA.

Glucagon-like peptide 1 (GLP-1) and glucagon-like peptide 1 derivatives (GLP-1 derivatives) are additional molecules that can be delivered therapeutically using the in vivo protein production and delivery system described in the present invention. GLP-1 derivatives include truncated derivatives GLP-1(7-37), GLP-1(7-36),

GLP-1(7-35) GLP-1(7-34) and other truncated
carboxy-terminal amidated derivatives and derivatives of
GLP-1 which have amino acid substitutions, deletions,
additions or other alterations (e.g., addition of a
5 non-amino acid component) which result in biological
activity or stability in the blood which is substantially
the same as that of a truncated GLP-1 derivative or
enhanced biological activity or stability in the blood
(greater than that of a truncated GLP-1 derivative). As
10 used herein, the term GLP-1 derivative includes all of
the above-described molecules. The term GLP-1 related
peptide, as used herein, includes GLP-1 and GLP-1
derivatives. GLP-1 derivatives, also known as
insulinotropins or incretins, are normally secreted into
15 the circulation by cells in the gastrointestinal tract.
In vivo studies have demonstrated that these peptides
function to stimulate insulin secretion and inhibit
glucagon secretion from the endocrine pancreas, as well
as increase insulin sensitivity in peripheral tissues
20 [Goke, R. et al. (1991) Eur. J. Clin. Inv. 21:135-144;
Gutniak, M. et al. (1992) New Engl. J. Med.
326:1316-1322]. Patients with non-insulin dependent
diabetes mellitus (NIDDM) are often treated with high
levels of insulin to compensate for their decreased
25 insulin sensitivity. Thus, the stimulation of insulin
release and the increase in insulin sensitivity by GLP-1
derivatives would be beneficial for NIDDM patients. Of
particular importance is the fact that the
insulinotropin-induced stimulation of insulin secretion
30 is strongly dependent on glucose levels, suggesting that
these peptides act in response to increases in blood

The present invention will now be illustrated by the following examples, which are not intended to be limiting in any way.

EXAMPLE 1. ISOLATION OF FIBROBLASTS

Human fibroblasts can be obtained from a variety of
10 tissues, including biopsy specimens derived from liver,
kidney, lung and skin. The procedures presented here are
optimized for the isolation of skin fibroblasts, which
are readily obtained from individuals of any age with
minimal discomfort and risk (embryonic and fetal fibro-
15 blasts may be isolated using this protocol as well).
Minor modifications to the protocol can be made if the
isolation of fibroblasts from other tissues is desired.

b. Isolation of Human Fascial Fibroblasts. Approximately 3 cm² tissue is placed into approximately 10 ml of wash solution (Hank's Balanced Salt Solution containing 100 units/ml penicillin G, 100 µg/ml streptomycin sulfate, and 0.5 µg/ml Fungisone) and subjected to gentle agitation for a total of three 10-minute washes at room

temperature. The tissue is then transferred to a 100 mm tissue culture dish containing 10 ml digestion solution (wash solution containing 0.1 units/ml collagenase A, 2.4 units/ml grade II Dispase).

5 Under a dissecting microscope, the skin is adjusted such that the epidermis is facing down. The fascial tissue is separated from the dermal and epidermal tissue by blunt dissection. The fascial tissue is then cut into small fragments (less than 1 mm²) and incubated on a
10 rotating platform for 30 min at 37°C. The enzyme/cell suspension is removed and saved, an additional 10 cc of digestion solution is added to the remaining fragments of tissue, and the tissue is reincubated for 30 min at 37°C. The enzyme/cell suspensions are pooled, passed through a
15 15-gauge needle several times, and passed through a Collector Sieve (Sigma) fitted with a 150-mesh screen. The cell suspension is centrifuged at 1100 rpm for 15 min at room temperature. The supernatant is aspirated and the disaggregated cells resuspended in 10 ml of nutrient
20 medium (see below). Fibroblast cultures are initiated on tissue culture treated flasks (Corning) at a density of approximately 40,000 cells/cm².

c. Isolation of Human Dermal Fibroblasts. Fascia is removed from skin biopsy or circumcision specimen as
25 described above and the skin is cut into small fragments less than 0.5 cm². The tissue is incubated with 0.25% trypsin for 60 min at 37°C (alternatively, the tissue can be incubated in trypsin for 18 hrs at 4°C). Under the
dissecting microscope, the dermis and epidermis are
30 separated. Dermal fibroblasts are then isolated as described above for fascial fibroblasts.

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d. Isolation of Rabbit Fibroblasts. The procedure is essentially as described above. Skin should be removed from areas that have been shaved and washed with a germicidal solution and surgically prepared using
05 accepted procedures.

EXAMPLE 2. CULTURING OF FIBROBLASTS

a. Culturing of Human Fibroblasts. When confluent, the primary culture is trypsinized using standard methods and seeded at approximately 10,000 cells/cm². The cells
10 are cultured at 37°C in humidified air containing 5% CO₂. Human fibroblast nutrient medium (containing DMEM, high glucose with sodium pyruvate, 10-15% calf serum, 20 mM HEPES, 20 mM L-glutamine, 50 units/ml penicillin G, and 10 µg/ml streptomycin sulfate) is changed twice weekly.

b. Culturing of Rabbit Fibroblasts. The cells are trypsinized and cultured as described for human fibro-
15 blasts. Rabbit fibroblast nutrient medium consists of a 1:1 solution of MCDB-110 (Sigma) with 20% calf serum and conditioned medium. Conditioned medium is essentially
20 human fibroblast nutrient medium (with 15% calf serum) removed from rabbit fibroblasts grown in mass culture for 2-3 days.

EXAMPLE 3. CONSTRUCTION OF A PLASMID (pXEP01) CONTAINING
25 THE HUMAN ERYTHROPOIETIN GENE UNDER THE
CONTROL OF THE MOUSE METALLOTHIONEIN PROMOTER

The expression plasmid pXEP01 has the hEPO gene under the transcriptional control of the mouse metallothionein (mMT) promoter. pXEP01 is constructed as
30 follows: Plasmid pUC19 (ATCC #37254) is digested with

KpnI and BamHI and ligated to a 0.7 kb KpnI-BgIII fragment containing the mouse metallothionein promoter [Hamer, D.H. and Walling, M., J. Mol. Appl. Gen., 1:273-288 (1982). This fragment can also be isolated by
05 known methods from mouse genomic DNA using PCR primers designed from analysis of mMT sequences available from Genbank; i.e. MUSMTI, MUSMTIP, MUSMTIPRM]. The resulting clone is designated pXQM2.

The hEPO gene was isolated by from a bacteriophage
10 lambda clone containing the entire hEPO gene. This bacteriophage was isolated by screening a human Sau3A-partial genomic DNA library (Stratagene) constructed in the lambda vector LAMBDA DASH with 0.77 kb fragment of the human gene. This 0.77 kb fragment was amplified from
15 human genomic DNA using the primers shown below in the polymerase chain reaction (PCR).

HUMAN EPO PCR PRIMERS:

- Oligo hEPO-1: 5'GTTTGCTCAGCTTGGTGCTTG (Seq. ID No. 1)
(positions 2214-2234 in the Genbank HUMERPA sequence)
- 20 Oligo hEPO-2: 5'TCAAGTTGGCCCTGTGACAT (Seq. ID No. 2)
(positions 2986-2967 in the Genbank HUMERPA sequence)

The amplified fragment, encompassing exons 4 and 5
of the human EPO gene, was radiolabelled and used to
25 screen the human genomic DNA library. Phage with a 5.4 kb HindIII-BamHI fragment containing the entire human EPO gene were assumed to contain the entire gene, based on published DNA sequence and restriction enzyme mapping

data [Lin, F-K., et al., Proc. Natl. Acad. Sci. USA,
82:7580-7584 (1985)].

05 A 4.8 kb BstEII-BamHI fragment (BstEII site is at
position 580 in Genbank HUMERPA sequence; the BamHI site
is 4.8 kb 3' of this site, outside of the sequenced
region) was isolated from the bacteriophage clone. The
purified fragment is made blunt-ended by treatment with
the Klenow fragment of E. coli DNA polymerase and ligated
to HincII digested pXQM2, which cuts in the pUC19-derived
10 polylinker adjacent to the 3' side of the subcloned mMT
promoter. One orientation, in which the ablated BstEII
site is proximal to the mMT promoter, was identified by
restriction mapping and designated pXEP01 (Figure 1).

15 EXAMPLE 4. TRANSFECTION OF PRIMARY AND SECONDARY
FIBROBLASTS WITH EXOGENOUS DNA AND A
SELECTABLE MARKER GENE BY ELECTROPORATION
AND MICROINJECTION

To prepare cells for electroporation, exponentially
growing or early stationary phase fibroblasts are
20 trypsinized and rinsed from the plastic surface with
nutrient medium. An aliquot of the cell suspension is
removed for counting, and the remaining cells are sub-
jected to centrifugation as described above. The super-
natant is aspirated and the pellet is resuspended in 5 ml
25 of electroporation buffer (20 mM HEPES pH 7.3, 137 mM
NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 6 mM dextrose). The
cells are recentrifuged, the supernatant aspirated, and
the cells resuspended in electroporation buffer con-
taining 1 mg/ml acetylated bovine serum albumin. The
30 final cell suspension contains approximately 3×10^6

cells/ml. Electroporation should be performed immediately following resuspension.

Supercoiled plasmid DNA is added to a sterile cuvette with a 0.4 cm electrode gap (Bio-Rad). The final
05 DNA concentration is generally at least 120 $\mu\text{g/ml}$. 0.5 ml of the cell suspension (containing approximately 1.5×10^6 cells) is then added to the cuvette, and the cell suspension and DNA solutions are gently mixed. Electro-
poration is performed with a Gene-Pulser apparatus
10 (Bio-Rad). Capacitance and voltage are set at 960 μF and 250-300 V, respectively. As voltage increases, cell survival decreases, but the percentage of surviving cells that stably incorporate the introduced DNA into their genome increases dramatically. Given these parameters, a
15 pulse time of approximately 14-20 mSec should be observed.

Electroporated cells are maintained at room temperature for approximately 5 min, and the contents of the cuvette are then gently removed with a sterile transfer
20 pipette. The cells are added directly to 10 ml of prewarmed nutrient media (as above with 15% calf serum) in a 10 cm dish and incubated as described above. The following day, the media is aspirated and replaced with 10 ml of fresh media and incubated for a further 16-24
25 hrs. Subculture of cells to determine cloning efficiency and to select for G418-resistant colonies is performed the following day. Cells are trypsinized, counted and plated; typically, fibroblasts are plated at 10^3 cells/10 cm dish for the determination of cloning efficiency and
30 at $1-2 \times 10^4$ cells/10 cm dish for G418 selection.

Human fibroblasts are selected for G418 resistance in medium consisting of 300-400 μ g/ml G418 (Geneticin, disulfate salt with a potency of approximately 50%; Gibco) in fibroblasts nutrient media (with 15% calf serum). Cloning efficiency is determined in the absence of G418. The plated cells are incubated for 12-14 days, at which time colonies are fixed with formalin, stained with crystal violet and counted (for cloning efficiency plates) or isolated using cloning cylinders (for G418 plates). Electroporation and selection of rabbit fibroblasts is performed essentially as described for human fibroblasts, with the exception of the nutrient media used. Rabbit fibroblasts are selected for G418 resistance in medium containing 1 mg/ml G418.

Fibroblasts were isolated from freshly excised human foreskins. Cultures were seeded at 50,000 cells/cm² in DMEM + 10% calf serum. When cultures became confluent fibroblasts were harvested by trypsinization and transfected by electroporation. Electroporation conditions were evaluated by transfection with the plasmid pCDNEO. A representative electroporation experiment using near optimal conditions (60 μ g of plasmid pCDNEO at an electroporation voltage of 250 volts and a capacitance setting of 960 μ Farads) resulted in one G418^r colony per 588 treated cells (0.17% of all cells treated), or one G418^r colony per 71 clonable cells (1.4%).

When nine separate electroporation experiments at near optimal conditions (60 μ g of plasmid pCDNEO at an electroporation voltage of 300 volts and a capacitance setting of 960 μ Farads) were performed, an average of one

G418^r colony per 1,899 treated cells (0.05%) was observed, with a range of 1/882 to 1/7,500 treated cells. This corresponds to an average of one G418^r colony per 38 clonable cells (2.6%).

5 Low passage primary human fibroblasts were converted to hGH expressing cells by co-transfection with plasmids pcDNEO and pXGH5 [Selden, R.F. et al., Mol. Cell. Biol., 6:3173-3179 (1986)]. Typically, 60 μ g of an equimolar mixture of the two plasmids were transfected at near
10 optimal conditions (electroporation voltage of 300 volts and a capacitance setting of 960 μ Farads). The results of such an experiment resulted in one G418^r colony per 14,705 treated cells.

hGH expression data for these and other cells
15 isolated under identical transfection conditions are
summarized below. Ultimately, 98% of all G418^r colonies
could be expanded to generate mass cultures.

	Number of G418 ^r Clones	
	Analyzed	154
20	Number of G418 ^r /hGH	
	Expressing Clones	65
	Average hGH Expression	
	Level	2.3 μg hGH/10 ⁶ Cells/24 hr
	Maximum hGH Expression	
25	Level	23.0 μg hGH/10 ⁶ Cells/24 hr

Stable transfectants also have been generated by electroporation of primary or secondary human fibroblasts with pXGH301, a DNA construct in which the neo and hGH genes are present on the same plasmid molecule (Example

3). For example, 1.5×10^6 cells were electroporated with 60 μ g pXGH301 at 300 volts and 960 μ Farads. G418 resistant colonies were isolated from transfected secondary fibroblasts at a frequency of 652 G418 resistant colonies per 1.5×10^6 treated cells (1 per 2299 treated cells). Approximately 59% of these colonies express hGH.

Primary and secondary human fibroblasts can also be transfected by direct injection of DNA into cell nuclei.

10 The ability of primary and secondary human foreskin fibroblasts to be stably transfected by this method has not been previously reported. The 8 kb HindIII fragment from plasmid RV6.9h (Zheng, H. et al., Proc. Natl. Acad. Sci. USA 88:18 8067-8071 (1991)) was purified by gel

15 electrophoresis and passage through an anion exchange column (QIAGEN Inc.). DNA at (10 μ g/ml) was injected into primary or secondary human foreskin fibroblasts using 0.1 μ m outer diameter glass needles. 41 G418^r clones were isolated after injection of 2,000 cells (1 in

20 49 starting cells).

hGH expressing clones were also generated by micro-injection. Plasmid pXGH301 (Figure 3) was linearized by ScaI digestion (which cuts once within the amp^r gene in the pUC12 backbone), purified by passage through an anion

25 exchange column (QIAGEN Inc.), and injected into secondary human foreskin fibroblasts using 0.1 μ m outer diameter glass needles. Several DNA concentrations were used, ranging from 2.5-20 μ g pXGH301/ml. Twenty G418 resistant clones were isolated after microinjection into

30 2,100 cells (1 in 105 starting cells). The fraction of G418^r cells, is approximately 1% of all cells treated.

Nine of 10 clones analyzed were expressing hGH, with average hGH expression being $0.6 \mu\text{g}/10^6$ cells/24 hr for clones isolated in this experiment, and 3 clones were expanded for studying long-term expression of hGH. All 3 were expressing hGH stably, with hGH still being produced through 33, 44, and 73 mpd for the 3 strains, respectively.

EXAMPLE 5. IN VITRO hEPO PRODUCTION BY TRANSFECTED
SECONDARY HUMAN AND RABBIT SKIN FIBROBLASTS

10 1. Human Skin Fibroblasts

Fibroblasts were isolated from freshly excised human skin fibroblasts and cultured in DMEM + 15% calf serum. Electroporation (250 volts, 960 μFarads) with 60 μg of an equimolar mixture of pCDNEO and pXEPO1 was performed on
15 passage 1 cells and treated cells were selected in G418-containing medium (300 $\mu\text{g}/\text{ml}$ G418). Colonies were isolated and expanded using standard methods. Data derived from an analysis of fifty-six individual clones is shown in Table 1 below. Cells were maintained in G418
20 (300 $\mu\text{g}/\text{ml}$ G418) in DMEM + 15% calf serum and subcultured at a seeding density of 10,000 cells/ cm^2 . Culture medium was changed 24 hr prior to harvesting the cells for passaging. At the time of passage, an aliquot of the culture medium was removed for hEPO assay and the cells
25 were then harvested, counted, and reseeded. hEPO concentration in the medium was determined using a commercially available ELISA (R & D Systems). hEPO levels are expressed as $\text{mU}/10^6$ cells/24 hr., and expression levels ranged from 69 to 55,591 $\text{mU}/10^6$ cells
30 /24 hr. 19% of all G418-resistant colonies expressed detectable levels of hEPO.

TABLE 1

hEPO EXPRESSION IN FIFTY-SIX INDEPENDENT
SECONDARY HUMAN FIBROBLAST CLONES ISOLATED BY
CO-TRANSFECTION WITH pcDNEO AND pXEPO1

5	hEPO Expression Level (mU/10 ⁶ cells/24 hr)	Number of Clones
	<1,000	10
	1,000-10,000	28
	10,000-50,000	17
10	>50,000	1

Clonally derived human fibroblasts isolated by co-transfection with pcDneo and pXEPO1 were analyzed for the glycosylation state of secreted hEPO. Media was collected from hEPO producing cells and immunoprecipitated with a mouse monoclonal antibody (Genzyme Corporation) specific for human erythropoietin. The immunoprecipitated material was subject to electrophoresis on a 12.5% polyacrylamide gel and transferred to a PVDF membrane (Millipore). The membrane was probed with the same anti-hEPO monoclonal antibody used for immunoprecipitation and was subsequently treated with an HRP-conjugated sheep anti-mouse IgG antisera (Cappel), followed by luminescent detection (ECL Western blotting detection kit; Amersham) to visualize hEPO through the production of a fluorescent product.

As shown in Figure 5A, a molecule with a molecular mass of approximately 34 kd reacts with an antibody specific for human erythropoietin. This is the expected size for naturally occurring, fully glycosylated human erythropoietin.

hEPO produced by transfected human fibroblast clones was further analyzed to determine if the secreted material had both N- and O-linked glycosylation characteristic of natural human erythropoietin isolated from urine or recombinant hEPO produced by chinese hamster ovary cells. Figure 5B shows a Western blot of the untreated cell supernatant (lane 1), the supernatant treated with endoglycosidase-F [(New England Nuclear); lane 2], the supernatant treated with neuraminidase [Genzyme]; (lane 3)], and the supernatant treated with O-glycanase [(Genzyme); (lane 4)]. Treatment with endoglycosidase-F results in a shift in molecular weight from 34 kd to approximately 27 kd. Treatment with neuraminidase results in a barely detectable shift in band position, while treatment with O-glycanase further shifts the size of the immunoreactive band down to approximately 18.5 kd. These results are indistinguishable from those obtained with natural human erythropoietin isolated from urine or recombinant hEPO produced by Chinese hamster ovary cells (Browne, J.K. et al., Cold Spring Harbor Symp. Quant. Biol. 51:693-702 (1986)).

2. Rabbit Fibroblasts

Fibroblasts were isolated from freshly excised rabbit skin and cultured in DMEM 10% calf serum. Electroporation (250 volts, 960 μ Farads) with 60 μ g of an

equimolar mixture of pcDNEO and pXEPO1 was performed and treated cells were selected in G418-containing rabbit fibroblast growth medium (1 mg/ml G418; Example 2). Colonies were isolated and expanded using standard methods, and the resulting secondary cell strains were analyzed for hEPO expression. Data derived from forty-nine independent rabbit fibroblast clones is shown in Table 2 below. Expression levels in these clones ranged from 43 to 2,900,000 mU/10⁶ cells/24 hr., and 72% of all G418-resistant clones expressed detectable levels of hEPO.

TABLE 2

hEPO EXPRESSION IN FORTY-NINE INDEPENDENT
SECONDARY RABBIT FIBROBLAST CLONES ISOLATED
BY CO-TRANSFECTION WITH pcDNEO AND pEEPO

<u>hEPO Expression Level</u> <u>(mU/10⁶ cells/24 hr)</u>	<u>Number of Clones</u>
<1,000	1
1,000-10,000	3
10,000-50,000	7
50,000-500,000	19
>500,000	19

EXAMPLE 6. CONSTRUCTION OF A PLASMID CONTAINING BOTH THE
HUMAN EPO GENE AND THE NEOMYCIN RESISTANCE
GENE

05 A 6.9 kb HindIII fragment extending from positions
11,960-18,869 in the HPRT sequence [Genbank entry
HUMHPRTB; Edwards, A. et al., Genomics, 6:593-608 (1990)]
and including exons 2 and 3 of the HPRT gene, is
subcloned into the HindIII site of pUC12. The resulting
clone is cleaved at the unique XhoI site in exon 3 of the
10 HPRT gene fragment and the 1.1 kb SalI-XhoI fragment
containing the neo gene from pMC1NEO (Stratagene) is
inserted, disrupting the coding sequence of exon 3. One
orientation, with the direction of neo transcription
opposite that of HPRT transcription was chosen and
15 designated pE3Neo. pE3neo has a unique XhoI site at the
junction of HPRT sequences and the 5' side of the neo
gene. pE3neo is cut with XhoI and made blunt-ended by
treatment with the Klenow fragment of E. coli DNA poly-
merase.

20 To insert the hEPO gene into the neo selection
plasmid pE3Neo, a 5.1 kb EcoRI-HindIII fragment was
isolated from plasmid pXEPO1 (Example 3; Figure 1). The
EcoRI site is located adjacent to the 5' side of the mMT
promoter, and the HindIII site is located 5.1 kb away, 3'
25 to the hEPO coding region. The purified Fragment is made
blunt-ended by treatment with Klenow fragment of E. coli
DNA polymerase and ligated to the XhoI digested and
blunt-ended pE3neo fragment described above. After
transformation into E. coli, a plasmid with one copy of
30 the mMT-hEPO fragment inserted into pE3neo was identified
by restriction enzyme analysis in which the hEPO gene is
transcribed in the same orientation as the adjacent neo

gene. This plasmid was designated pE3neoEPO. In addition to allowing direct selection of hEPO expressing G418^r clones, this fragment may also be used in gene targeting to direct the integration of the hEPO gene to
05 the human HPRT locus.

EXAMPLE 7. ISOLATION OF HUMAN FIBROBLAST CLONES
EXPRESSING hEPO GENE AND A SELECTABLE
MARKER (pE3neoEPO)

Fibroblasts were isolated from freshly excised human
10 skin fibroblasts and cultured in DMEM + 15% calf serum. Electroporation (250 volts, 960 μ Farads) with 60 μ g of supercoiled pE3neoEPO was performed on passage 1 cells and treated cells were selected in G418-containing medium (300 μ g/ml G418). Colonies were isolated and expanded
15 using standard methods. Data derived from an analysis of twenty-six individual clones is shown in Table 3 below. Cells were maintained in G418 (300 μ g/ml G418) in DMEM + 15% calf serum and subcultured at a seeding density of 10,000 cells/cm². Culture medium was changed 24 hr prior
20 to harvesting the cells for passaging. At the time of passage an aliquot of the culture medium was removed for hEPO assay and the cells were then harvested, counted, and reseeded. hEPO concentration in the medium was determined using a commercially available ELISA (R and D
25 Systems). hEPO levels are expressed as mU hEPO/10⁶ cells/24 hr, and expression levels ranged from 240 to 961,620 mU/10⁶ cells/24 hr. 89% of all G418-resistant clones expressed detectable levels of hEPO.

TABLE 3

hEPO EXPRESSION IN TWENTY-SIX INDEPENDENT
SECONDARY HUMAN FIBROBLAST CLONES ISOLATED
BY TRANSFECTION WITH pE3neo-EPO

<u>hEPO Expression Level</u> <u>(mU/10⁶ cells/24 hr)</u>	<u>Number of Clones</u>
<1,000	2
1,000-10,000	2
10,000-50,000	9
50,000-500,000	12
>500,000	1

hEPO expressing human fibroblast clones are also isolated by electroporation with 60 μ g of HindIII digested pE3neoEPO. hEPO expressing rabbit fibroblast clones are isolated using plasmid pE3neoEPO under identical transfection conditions, with the exception that rabbit fibroblast clones are selected in rabbit fibroblast growth medium (Example 2) containing 1 mg/ml G418.

10 EXAMPLE 8. ISOLATION OF TRANSFECTANTS IN THE ABSENCE OF
SELECTION

The high frequency of transfection in human fibroblasts (greater than 1% stable transfectant per clonable cell; Example 4) indicates that it should be possible to isolate cell clones that have stably incorporated exogenous DNA without the use of a selective agent. Stable transfection of primary fibroblasts with the

plasmid pXEPO1 should render recipient fibroblasts capable of secreting human erythropoietin into the surrounding medium. Therefore, an ELISA for hEPO (or for any expressed protein of therapeutic interest) can be used as a simple and rapid screen for transfectants. Alternatively, it should be possible to determine the true frequency of stable integration of exogenous DNA using a screening method such as PCR which does not necessarily rely on expression of transfected DNA.

1. Primary Human Fibroblasts

Approximately 2.0×10^6 human cells that were freshly dissociated from tissue are electroporated with 60 μ g of pXEPO1 at 300 volts, 960 μ Farads. Cells are plated immediately in a 100 mm tissue culture dish containing 10 ml of prewarmed medium and incubated at 37°C in a humidified 5% CO₂ atmosphere. Two days following transfection, 5×10^3 cells are subcultured into a 24 well cloning plate (Bellco Glass Co.). Each well of the 24 well plate contained 16 smaller wells (384 wells/plate). Eight days after plating into the 24 large wells, media is screened for hEPO expression via ELISA. A second, confirming assay, is done in which media from wells exhibiting hEPO expression is aspirated out, replaced with fresh media and assayed for hEPO 24 hours later. Colony counts at this stage should reveal approximately 10 colonies per large well.

Individual colonies in each of the 16 small wells within one of the hEPO-positive larger wells are trypsinized and transferred to wells of a 96 well plate. Three days later each of those wells are assayed for hEPO

2. Primary Rabbit Fibroblasts

20 Direct injection of DNA into cell nuclei is another method for stably transfecting cells. The ability of primary and secondary human foreskin fibroblasts to be stably transfected by this method is described in Example 4, but has not been previously reported in the literature. The 13.1 kb HindIII fragment from plasmid pE3neoEPO is purified by gel electrophoresis and passed through an anion exchange column (QIAGEN Inc.). This fragment contains the human EPO and bacterial neo genes, flanked on both sides with human HPRT sequences. DNA at (10 μ g/ml) is injected into primary or secondary human foreskin fibroblasts using 0.1 μ m diameter glass needles.

EXAMPLE 9. STABLE TRANSFECTION OF PRIMARY HUMAN FIBROBLASTS BY MICROINJECTION

20 Direct injection of DNA into cell nuclei is another method for stably transfecting cells. The ability of primary and secondary human foreskin fibroblasts to be stably transfected by this method is described in Example 4, but has not been previously reported in the literature. The 13.1 kb HindIII fragment from plasmid pE3neoEPO is purified by gel electrophoresis and passed through an anion exchange column (QIAGEN Inc.). This fragment contains the human EPO and bacterial neo genes, flanked on both sides with human HPRT sequences. DNA at (10 μ g/ml) is injected into primary or secondary human foreskin fibroblasts using 0.1 μ m diameter glass needles.

G418^r clones are isolated approximately 12-14 days after injection. Alternatively, the total HindIII digest of pE3neoEPO, as well as linearized or supercoiled pE3neoEPO may be injected to isolate hEPO expressing cells.

05 EXAMPLE 10. EXPRESSION OF BIOLOGICALLY ACTIVE HUMAN
ERYTHROPOIETIN IN MICE

The mouse provides a valuable system to study implants of genetically engineered cells for their ability to deliver therapeutically useful proteins to an
10 animal's general circulation. The relative immune-incompetence of nude mice allow xenogeneic implants to retain biologic function and may allow certain primary and secondary rabbit fibroblasts to survive in vivo for extended periods.

15 For implantation of cells into the subrenal capsule, mice are given intraperitoneal injection of Avertin at a dose of 0.0175 ml/g body weight. The kidney (generally the left kidney) is approached through an 8-10 mm incision made approximately 3 mm below the rib cage. The
20 skin, abdominal musculature, peritoneum, and peri-renal fascia are retracted to expose the kidney. A small forcep is used to pull the kidney out of the abdominal cavity. A 27-gauge hypodermic needle is used to make a small opening in the renal capsule. Using a 20-gauge
25 I.V. catheter, cells to be implanted (typically 3 million cells in a volume of 5-10 μ l) are withdrawn into a 1 ml syringe and slowly ejected under the renal capsule. Care is taken to ensure that the cells are released distal to the opening in the renal capsule. The incision is closed
30 with one staple through the musculature and the skin. Blood is collected after placing the mouse in a large

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immunoreactive hEPO was readily detectable in the blood of implanted animals (the sensitivity of the hEPO ELISA has been determined to be 2 mU/ml by the kit's manufacturer (R and D Systems) and endogenous mouse EPO shows no cross-reactivity with the antibodies used in the ELISA kit). hEPO levels in the implanted animals dropped gradually, from 29 to 9 mU/ml, from days 7 to 63 post-implantation.

This Example clearly demonstrates that normal skin fibroblasts that have been genetically engineered to express and secrete hEPO can: 1) survive in vivo to deliver hEPO to an animals systemic circulation for up to 2 months, and 2) the hEPO produced is biologically functional, serving to prevent the drop in hematocrit observed in the frequently bled control animals, and resulting in a net increase in HCT even when animals were challenged with a bleeding schedule that produces an anemic response in control animals.

EXAMPLE 11. EXPRESSION OF GLP-1(7-37) FROM SECONDARY
HUMAN SKIN FIBROBLASTS STRAINS AFTER
TRANSFECTION WITH A GLP-1(7-37)
EXPRESSION PLASMID

The portion of GLP-1 from amino acid residues 7 to 37 [GLP-1(7-37); encoded by base pairs 7214 to 7306 in Genbank sequence HUMGLUCG2] has been demonstrated to have insulinotropin activity in vivo. Plasmid pXGLP1 is constructed such that the GLP-1(7-37) moiety is fused at its N-terminus to a 26-amino acid signal peptide derived from human growth hormone for efficient transport through the endoplasmic reticulum. The fusion protein is cleaved

immediately C-terminal to residue 26 prior to secretion,
such that the secreted product consists solely of
residues 7-37 of GLP-1. Expression of the signal
peptide: GLP-1(1-37) fusion protein is controlled by the
05 mouse metallothionein promoter.

Plasmid pXGLP1 is constructed as follows: Plasmid
PXGH5 [Selden, R.F. et al., Mol. Cell. Biol. 6:3173-3179
(1986)] is digested with SmaI and ligated to a
double-stranded oligonucleotide containing a BgIII site
10 (BgIII linkers; New England Biolabs). The ligated
product is digested with BgIII and EcoRI and the 0.32 kb
fragment corresponding to the 3'-untranslated region of
the human growth hormone gene is isolated (with a BgIII
linker attached to the SmaI site lying at position 6698
15 in Genbank entry HUMGHCSA). The hGH fragment can also be
isolated by known methods from human genomic DNA using
PCR primers designed to amplify the sequence between
positions 6698 to 7321 in Genbank entry HUMGHCSA. A 1.45
EcoRI-BgIII fragment containing the mouse metallothionein
20 (mMT) promoter [Hamer, D.H. and Walling, M., J. Mol.
Appl. Gen., 1:273-288 (1982)] is next isolated. The
mouse metallothionein promoter may be isolated by known
methods from mouse genomic DNA using PCR primers designed
from analysis of mMT sequences available from Genbank
25 (i.e. Genbank entries MUSMTI, MUSMTIP, and MUSMTIPRM).
Plasmid pUC19 (ATCC #37254) is digested with EcoRI and
treated with bacterial alkaline phosphatase. The treated
plasmid is ligated with the hGH and mMT fragments
described above. The resulting plasmid has a single copy
30 of each the mouse metallothionein promoter and the
3'untranslated region of hGH joined at a BgIII site.

This plasmid, designated pX1 is digested with BgIII and the full-length linear product is purified by gel electrophoresis.

05 Oligonucleotides 11.1 and 11.2 are used to amplify a DNA sequence encoding amino acids 7-37 of GLP-1 from human genomic DNA by PCR. The amplified product (104 bp) is purified and mixed with pXGH5 and oligonucleotides 11.2, 11.3, 11.4, and 11.5 and subject to PCR.

10 Oligonucleotides 11.3 and 11.4 are complementary and correspond to the desired junction between the hGH signal peptide and GLP-1 amino acid residue 7. The 500 base pair amplification product contains 5'-untranslated, exon 1, intron 1, and part of exon 2 sequences from hGH (nucleotides 5168 to 5562 in Genbank entry HUMGHCSA)
15 fused to a sequence encoding GLP-1 residues 7-37 followed by a stop codon. The fragment, by design, is flanked on both ends by BamHI sites.

The fragment is cleaved with BamHI and ligated to the BgIII digest of pX1 described above. Ligation
20 products are analysed to identify those with one copy of the hGH-GLP-1(7-37) fusion product inserted at the BgIII site separating the mMT promoter and the 3'-untranslated hGH sequence in pX1, such that GLP-1 residue 37 is distal to the mMT promoter.

25 OLIGONUCLEOTIDES FOR AMPLIFICATION OF hGH-GLP-1(7-37)
FUSION GENE

11.1 5'CATGCTGAAG GGACCTTTAC CAGT (Seq. ID No. 3)

11.2 5'TTGGATCCTT ATCCTCGGCC TTTCACCAGC CA (Seq. ID No. 4)

BamHI

11.4 5'ACTGGTAAAG GTCCCTTCAG CATGGGCACT GCCCTCTTGA AGCC
(Seq. ID No. 6)

11.6 5' TTGGATCCTT ATCGGCC TTTCACCAGC CA (Seq. ID No. 8)
BamHI

Alternatively, the small sizes of the signal peptide and GLP-1 moieties needed allow complete fusion genes to be prepared synthetically. DNA encoding the signal peptides of the LDL receptor (amino acid residues 1-21), preproglucagon (amino acid residues 1-20), or human growth hormone (amino acid residues 1-26) may be synthesized by known methods and ligated in vitro to similarly synthesized DNA encoding amino acids 7-37 or 7-36 of GLP-1 (followed immediately by a stop codon). The sequences necessary to design and synthesize these molecules are readily available in Genbank entries HUMLDLR01 (human LDL receptor), HUMGLUCG2 (human GLP-1 and glucagon sequences), and HUMGHCSA (human growth hormone). The ligated product may be inserted into a suitable mammalian expression vector for use in human fibroblasts. Plasmid pMSG (Pharmacia LKB Biotechnology, Piscataway, NJ) is suitable for this purpose, having 5' and 3'untranslated sequences, a splice site, a polyA addition site, and an enhancer and promoter for use in human skin fibroblasts. Alternatively, the ligated product may be synthesized with an appropriate 5'-untranslated sequence and inserted into plasmid pX1 described above.

05 A second insulintropic GLP-1 derivative,
GLP-1(7-36), can be expressed by substituting
oligonucleotide 11.6 for oligonucleotide 11.2 described
above. All subsequent cloning operations described above
for construction of pXGLP1 are followed, such that the
final product is lacking the C-terminal glycine residue
characteristic of GLP-1(7-37). Alternatively, this
terminal glycine residue may be removed in vivo by the
activity of a peptidyl-glycine alpha-amidating enzyme to
10 produce the insulintropic GLP-1(7-36) amide.

Plasmid pXGLP1 is co-transfected into primary human
skin fibroblasts with plasmid pCDNEO exactly as described
for pXEPO1 and pCDNEO in Example 5. Clones are selected
in G418 containing medium, transferred to 96-well plates,
15 and assayed for GLP-1(7-37) activity or immunoreactivity
in cell supernatants. GLP-1(7-37) activity is determined
by incubation of cell supernatants with rat insulinoma
RINm5F cells and measuring the ability of the
supernatants to induce insulin secretion from these cells
20 using a commercially available insulin radioimmunoassay
(Coat-a-Count Insulin, DPC, Los Angeles, CA).
GLP-1(7-37) antigen is determined using a commercially
available antisera against GLP-1 (Peninsula Laboratories,
Belmont, CA). GLP-1(7-37) positive clones are expanded
25 for implantation into nude mice as described in Example
10 and blood samples are taken to monitor serum human
GLP-1(7-37) levels.

In vivo activity is monitored in fasting animals by
determining the insulinogenic index after intraperitoneal
30 injection of glucose (1 mg glucose per gram of body
weight). Typically, implanted and non-implanted groups

of 32 mice are fasted overnight, and 28 are injected with glucose. After injection, the 28 mice are arbitrarily assigned to seven groups of four, and blood sampling (for serum glucose and insulin) is performed on each group at 5, 10, 20, 30, 45, 60, or 90 minutes post-injection, with the non-glucose injected group serving as a fasting control. Increases in the postinjection insulinogenic index (the ration of insulin to glucose in the blood) in animals receiving GLP-1(7-37) expressing cells over non-implanted animals provides in vivo support for the insulinotropic activity of the expressed peptide.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using not more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

Statement Regarding Correspondence of Sequence Information (Paper Copy and Disk)

A sequence listing in computer readable form and in paper form are being filed with this application. As required by 37 C.F.R. 1.821 (f), Applicants' Attorney hereby states that the content of the Sequence Listing in paper form and of the computer readable form of the "Sequence Listing" are the same.